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# STUDIES ON BEEF SARCOPLASMIC PROTEINS

by

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# Untersuchungen über die Eiweißstoffe des Rindersarkoplasmas


## Zusammenfassung

Die Sarkoplasma - Eiweißstoffe des Rinderskelettmuskels wurden unter Verwendung von analytischem Ultrazentrifugieren und Gel-Filtration untersucht.

Es wurden fünf sedimentierende Eiweiß-Komponenten aufgelöst, deren Sedimentationskoeffizienten $S_{20,w}$ 8,6 7,4 4,0 5,2 und 2,1 S betrugen Die Eiweißstoffe wurden in fünf Fraktionen mit verschiedenen Molekulargewichten mittels Gel-Filtration auf Sephadex G-100 Säulen getrennt.

Die Eiweiß-Komponenten wurden mittels Ultrazentrifugieren und Stärke-Gel-Elektrophorese untersucht und charakterisiert. Die durchschnittlichen Sedimentationskoeffizienten der Eiweiß-Komponenten ($S_{20,w}$) waren 9,0 7,7 5,5 4,1 und 2,0 S.

Die Sedimentationsergebnisse werden mit veröffentlichten Angaben über die Sedimentationskoeffizienten und Molekulargewichte der aus Säugetierskelettmuskel isolierten Eiweißstoffen verglichen. Die einzelnen Eiweißstoffe in den Sephadex-Fraktionen wurden aufgrund dieses Vergleiches vorläufig identifiziert.

# STUDIES ON BEEF SARCOPLASMIC PROTEINS

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## ABSTRACT

The sarcoplasmic proteins of bovine skeletal muscle have been studied using analytical ultracentrifugation and gel filtration.

Five sedimenting protein components were resolved with sedimentation coefficients $S_{20,w}$ 8.6, 7.4, 5.2, 4.0, and 2.1S. The proteins have been separated into five fractions differing in molecular weight using gel filtration on G100 Sephadex columns.

The protein fractions have been studied and characterised by ultracentrifugation and starch gel electrophoresis. They have mean sedimentation coefficients ($S_{20,w}$) of 9.0, 7.7, 5.5, 4.1, and 2.0S.

The sedimentation results are compared with published data on the sedimentation coefficients and molecular weights of proteins which have been isolated from mammalian skeletal muscle. The individual proteins present in the Sephadex fractions have been tentatively identified on the basis of this comparison.


## INTRODUCTION

The sarcoplasmic proteins are obtained by extracting skeletal muscle with low ionic strength buffers - circa 0.1 - which do not bring the myofibrillar (structural) proteins into solution. Studies of the physicochemical properties of whole sarcoplasm from mammalian skeletal muscle tissues are few, and there has been little attempt at systematic fractionation of the proteins.

Sarcoplasmic extracts of mammalian muscle contain a large number of proteins including the pigment protein myoglobin, and the enzymes of the glycolytic cycle (which during life provide the energy required for muscular contraction from the breakdown of glycogen). It has been suggested that there are as many as 50 proteins present in sarcoplasm.(1) Sarcoplasmic extracts have, consequently, often been used as the starting point in methods for preparing the enzymes associated with the glycolytic cycle. A number of enzymes have in fact been isolated in crystalline form from such extracts, by either salting out with $(NH_4)_2SO_4$ at constant pH, variation of pH in the salting out region; precipitation with organic solvents in the salting in region; or by preparative starch block electrophoresis (2).

Analytical ultracentrifuge studies of whole sarcoplasm from skeletal muscle are limited. The earliest reported work was in 1934 by Deuticke (3) who studied rabbit and frog muscle extracts. He found two sedimenting

components of 1.1 and 7.6S in frog muscle and two components of 5.5 and 7.7S in rabbit muscle. Gralen (4) in 1939 showed that myogen A, a sarcoplasmic fraction of rabbit muscle, had a sedimentation coefficient of 7.86 S. The next reported study was in 1949 by Amberson et al (5) who found two components of 5.8 and 8.8 S in rabbit muscle. Kronman, Weinberger and Winterbottom in 1960 , published the only recorded work on bovine muscle.(6) They described three sedimenting components with sedimentation coefficients 2.3, 4.7, and 6.8 S.

Fish muscle has been studied rather more extensively by Hamoir and co-workers (7). They have shown fish to be quite different from mammalian skeletal muscle. In general three peaks are observed with sedimentation coefficients 1.3 - 1.5, 4.5 - 5.0 and 6.5 - 7.2 S. The slow sedimenting peak occurs only in the muscles of fish and frog. It amounts to 50% of the sedimenting material of carp muscle.

The purpose of our more recent work has been to develop methods for the identification and fractionation of the complex mixture of sarcoplasmic proteins from bovine skeletal muscle, so that the changes occurring in the proteins during the post mortem period and during meat storage can be studied, and we hope elucidated, in more detail.

We have shown that five distinct peaks can be resolved in sedimentation diagrams of bovine skeletal muscle sarcoplasmic extracts. The components have been separated and isolated on a preparative scale by gel filtration through Sephadex columns. Ultracentrifugal analysis showed that there was no gross heterogeneity in the Sephadex fractions. Tentative identification of some of the components has been made by comparison with published data on the sedimentation characteristics of crystalline enzymic proteins isolated from sarcoplasmic extracts.

MATERIALS AND METHODS

Samples of bovine longissimus dorsi muscle were obtained fresh within a few hours of death from a local slaughterhouse. The muscle was trimmed of external fat and connective tissues, and homogenised with 0.04M potassium phosphate buffer pH 7.4 in an MSE Homogeniser at high speed for 1 min (using 5 ml buffer per 1 g muscle tissue ) (8).

The extract was centrifuged at 6000 rpm (5,800 g) at 0° for 30 minutes in a Servall RC2 refrigerated centrifuge to remove the fibres and stroma, followed by centrifuging at 13,000 rpm (20,000 g) for 30 min. to remove any further particulate material (9). Any fatty material floating on the surface after centrifuging was removed by filtration of the extract. The resultant clear solution contained the soluble sarcoplasmic proteins.

The extracts were used immediately or lyophilized to form a stock sample, aliquots of which were redissolved prior to analysis as required.

GEL FILTRATION (Sephadex)

G25

Sephadex G25, bead form, was equilibrated with 0.04M potassium phosphate buffer for at least 24 hours before use. 20 x 2 cm columns were prepared in the usual manner. 4 mls of the crude sarcoplasmic extract containing about 100 mg of protein was fractionated into high ($>4,000$) and low ($<4,000$) molecular weight fractions, by eluting with 0.04 M potassium phosphate buffer pH 7.4, The first peak containing the sarcoplasmic proteins was lyophilized in preparation for further fractionation on G100 columns.

G100

Sephadex G100, bead form was equilibrated by suspension in 0.04 M potassium phosphate buffer pH 7.4 for 4-5 days at room temperature before use. 150 x 1 cm columns were prepared. The column was surrounded by a water jacket through which water maintained at 15°C was circulated. The columns were packed either under full hydrostatic head or at the pressure to be used during the experiment. Care was taken not to occlude air bubbles when the Sephadex suspension was poured into the column. After a 10 cm high bed level had formed, the clip at the base of the column was opened and flow of buffer commenced. When the column had reached the desired bed height the buffer reservoir was connected and buffer circulated continuously throughout the life of the column. The flow rate however gradually decreased with repeated use. To overcome this, the upper 1-2 cm of Sephadex was removed and replaced with freshly equilibrated Sephadex before each run. This upper layer contained the lipoprotein and other materials which could not enter the column and remained at the top of the column as a narrow band.

The lyophilized protein peak from the G25 separation was redissolved in 1 ml distilled water giving a sample containing about 100 mg/ml protein. The whole of the 1 ml sample was applied to the 150 x 1 cm G100 Sephadex column. The protein solution, clearly visible due to the red colour of the myoglobin, was allowed to sink into the gel and then washed into the column with small quantities of buffer before the reservoir was connected. Elution with 0.04 M potassium phosphate buffer pH 7.4 was carried out under a hydrostatic head of about 20 cm; the

hydrostatic head being adjusted to give the required flow rate - optimum about 6 ml per hour. A slow flow rate is essential for maximum resolution. The red coloured myoglobin was a useful index of column efficiency, as it indicated when there was a bad application of the sample - (shown by streaking) or faulty packing - (shown by a tendency of the protein to migrate down the sides of the column).

The effluent was collected as 1 ml fractions in a Locarte Fraction Collector using a drop counter. The tubes were diluted to 4 mls with buffer and monitored manually at 280 mμ in silica cells in a Unicam SP 500 Spectrophotometer. The tubes corresponding to each peak were lyophilized and stored at -20°C until required for further analysis.

ULTRACENTRIFUGATION

Ultracentrifugal analyses were carried out in a Spinco Model E Analytical Ultracentrifuge equipped with an RTIC unit. Schlieren optics were used throughout this work. All runs were carried out at 59,780 rpm and 20°C. The lyophilized samples were dialysed against buffer for 24-48 hrs. at 2°C, the buffer being constantly stirred using a magnetic stirrer, before ultracentrifugal analysis. Sedimentation coefficients were evaluated in the conventional manner (10), and corrected for the influence of solvent viscosity and density (11).

Concentration determinations were carried out using a filled epon double sector cell at 42,040 rpm at 20°C.

STARCH GEL ELECTROPHORESIS

Starch gel electrophoresis was carried out in borate buffer pH 8.6, using the procedures previously described (12).

RESULTS AND DISCUSSION

Ultracentrifuge studies of bovine sarcoplasmic extracts in 0.04 M K phosphate buffer pH 7.4 showed that five sedimenting components could be resolved. They had sedimentation coefficients extrapolated to infinite dilution of 8.6, 7.4, 5.2, 4.0 and 2.1 S. The major components are the 7 and 5 S peaks. The same number of components with the same sedimentation coefficients have been identified in all the samples of muscle examined. Qualitatively the Schlieren diagrams were very similar although there were variations in the amounts of the components from different animals (i.e. the area under the Schlieren curve showed some variation).

The results indicate the existence of five molecular weight groups in bovine sarcoplasmic extracts. The previous studies on bovine sarcoplasm showed three sedimenting species, with unextrapolated sedimentation coefficients 6.8, 4.7, and 2.3 S (6). These values are in satisfactory agreement with our results for the components of $S_{20,w}$ 7.4, 5.2, and 2.1 S.

The slowest moving component of 2.1S has a dark red boundary running through the centre of the peak; this shows that the component must be myoglobin.

Examination of the samples of whole sarcoplasm by starch gel electrophoresis showed all the usual resolvable components (12), (14). Here again variations from animal to animal were detected.

The demonstration of distinct sedimenting groups in the sarcoplasm extracts prompted us to investigate the possibility of separating the proteins by gel filtration using the cross-linked polydextran Sephadex.

Gel filtration uses a molecular sieving effect to separate macromolecules on a basis of their size. The first fractionation of the sarcoplasmic extracts on G25, separates the sarcoplasmic proteins (first peak) from the low molecular weight constituents of the sarcoplasm. G25 Sephadex has an exclusion limit of 4,000; that is molecules of molecular weight greater than 4,000 pass straight through the column since they cannot enter the gel pores, whereas molecules of molecular weight less than 4,000 diffuse into the Sephadex grains and are retarded during the elution. This can be seen in Figure 1 which shows the 280 and 260 mμ absorbance plotted against elution volume (fraction number). The low molecular weight peak includes the nitrogen bases - nucleotides such as ATP and its breakdown products - present in muscle tissue, and the free amino acids and small peptides such as carnosine and anserine. The 260 mμ absorption of the second peak is greater than the 280 mμ; this is an indication of the presence of the nucleotides, which have their absorption maximum about 260 mμ. The nucleotide bases have an appreciable residual absorption at 280 mμ, consequently if they are not removed they severely interfere with the monitoring of protein elutions by scanning in the ultraviolet region of the spectrum. The gel filtration through G25 columns enables us to overcome this difficulty in our subsequent analyses. Dialysis can also be used to remove the low molecular weight nucloetides, but gel filtration has the advantages of speed, the fractionation is complete, and the separation can be monitored. The purity of the protein can be assessed by the ratio of the 280/260 mμ

absorption (13). If this ratio is about 1.4 it corresponds to about 99.% protein. It can be seen that the first peak of the G25 separation is therefore almost pure protein.

The first peak of a G25 Sephadex separation gives an ultracentrifuge diagram identical to the unsieved sarcoplasmic extract with five clearly distinguishable peaks. Their uncorrected sedimentation coefficients were 7.4, 6.4, 5.0, 3.5 and 1.9 S. The usual protein components were also identified when the G25 peak was examined by starch gel electrophoresis i.e. it showed the same characteristics as whole sarcoplasm.

When the G25 protein peak was applied to a G100 Sephadex column which has an exclusion limit about 150,000, five components were resolved as shown in Figure 2. The extinctions at 280 and 260 mu are plotted against the elution volume. The peaks have been numbered $V_1$, $V_2$, $V_3$, $V_4$, $V_{5A}$, $V_{5B}$. $V_{5B}$ has a distinct red colour and can be identified from its spectrum as oxy-myoglobin. A coloured component also emerges during the latter part of $V_4$. The fractions corresponding to each of the peaks were pooled as indicated on the diagram and freeze dried prior to analysis by analytical ultracentrifugation and gel electrophoresis.

An excellent linear correlation has been shown to exist between the logarithm of the molecular weight of a globular protein and the ratio of its elution volume $V_e$ to the void volume $V_o$ of the Sephadex G100 column (15). Using this relationship we can estimate the molecular weight corresponding to the peak maxima of our components. The estimated molecular weights of the components - the mean of triplicate determinations - are given in Table 1. These are greater than 150,000, 110,000, 79,000, 52,000 and 14,000.

Table 1. Volume ratio and estimated molecular weight of G100 fractions

| | Volume ratio $V_e/V_o$ | | | | Molecular weight M x $10^{-4}$ | | | |
|---|---|---|---|---|---|---|---|---|
| Experiment No. | I | II | III | Mean | I | II | III | Mean M |
| G100 fraction | | | | | | | | |
| $V_1$ | 1.00 | | | | | | | 15-18 |
| $V_2$ | 1.18 | 1.13 | 1.16 | 1.16 | 10.4 | 11.6 | 10.8 | 10.9 |
| $V_3$ | 1.29 | 1.29 | 1.32 | 1.30 | 8.1 | 8.1 | 7.6 | 7.9 |
| $V_4$ | 1.47 | 1.48 | 1.51 | 1.49 | 5.4 | 5.3 | 4.9 | 5.2 |
| $V_{5A}$ | 1.99 | 1.85 | 1.91 | 1.88 | 1.6 | 2.3 | 2.0 | 2.1 |
| $V_{5B}$ | | 2.06 | 2.09 | 2.07 | | 1.4 | 1.4 | 1.4 |

The agreement between the estimated molecular weight of myoglobin-14,000 and the published value of 17,000 is reasonable, especially when it emerges as the last component of a complex fractionation. The other coloured component which emerges in $V_4$ has an estimated molecular weight of 35,000. It cannot be hemoglobin M.W. 67,000. It is very possibly a subunit of hemoglobin, which is known to dissociate in very dilute solutions into subunits of approximately 34,000 M.W. The hemoglobin will arise from the residual blood in muscle.

The percentage composition of the fractionated proteins based on their 280 mμ absorption is given in Table 2.

Table 2. Percentage composition of eluant from G100 columns (estimated from 280 mμ absorption)

| G100 Fraction | Experiment | | |
|---|---|---|---|
| | I | II | III |
| $V_1$ | 15.5 | 17.5 | 16.0 |
| $V_2$ | 26.8 | 33.9 | 34.5 |
| $V_3$ | 23.0 | 13.5 | 14.4 |
| $V_4$ | 9.8 | 10.0 | 9.8 |
| $V_5$ | 24.9 | 25.0 | 25.2 |
| $V_{5A}$ | | 4.8 | 4.4 |
| $V_{5B}$ | | 20.2 | 20.8 |

The reproducibility between different experiments can be seen to be quite satisfactory. The slight difference between $V_2$ and $V_3$ in the first experiment can be accounted for by the differing flow rates of the eluant through the column. The separation on G100 Sephadex does not yield individual proteins, but separates them into convenient groups of similar size and shape. This offers a convenient method of following changes in the molecular size as a result of protein changes post mortem.

Sedimentation analyses of the individual fractions showed that the fractions were relatively uncontaminated by neighbouring peaks, considering that the extract had only been passed once through the G100 column. The broadness of the peaks however indicated that the fractions were not homogenous in the sense of having only one protein constituent.

The ultracentrifuge results are summarised in Table 3.

Table 3. Ultracentrifugal analysis of G100 fractions

| G100 fraction | Main ultracentrifuge component | Minor ultracentrifuge component | Sedimentation coefficient S | Estimated concentration (g/100 ml) | % composition |
|---|---|---|---|---|---|
| $V_1$ | $P_1$ | | 9.21 | 0.10 | 3.0 |
| $V_2$ | $P_2$ | $P_1$ | 6.70 | 1.20 | 35.7 |
| $V_3$ | $P_3$ | $P_2$ | 4.69 | 1.21 | 36.0 |
| $V_4$ | $P_4$ | | 3.62 | 0.34 | 10.1 |
| $V_5$ | $P_5$ | | 1.84 | 0.51 | 15.1 |

Table 3 shows the uncorrected sedimentation coefficients for the main component in each fraction, 9.2,6.7,4.7,3.6 and 1.8 S, together with the protein concentration and percentage composition determined from double sector cell analysis using the diffusate as reference solvent and measuring the area under the Schlieren curve using a planimeter. The percentage composition shown in the final column of the Table has some marked differences from the calculated from the 280 mμ absorbance after gel filtration through G100 Sephadex. It reflects the difference between the two indices of protein concentration -refractive index in the ultracentrifuge and ultraviolet absorption in the elution. The refractive index determination is the more general of the two, since most proteins both globular and fibrous have a specific refractive increment close to 0.00186 for a 1% solution, whereas the aromatic amino acid content and hence the 280 mu extinction coefficient of proteins can vary between very wide limits.*

* The Sephadex components were studied at four dilutions to enable the sedimentation coefficients to be extrapolated to infinite dilution. This gave $S_{20,w}$ values of 9.0,7.7,5.5,4.1, and 2.0 S. It can be seen that the $S_{20,w}$ values of the individual G100 peaks closely resemble those of the unfractionated whole sarcoplasmic extract.

The G100 fractions were not electrophoretically homogenous when examined by starch gel electrophoresis. The extensive fractionation which had occurred as a result of the gel filtration was quite evident, since only certain discrete protein components, separated into well defined bands, appear in each fraction. Since the fractions have been shown to be very similar with respect to molecular weight and size by the gel filtration and ultracentrifugal analysis, the essential difference between the components resolved by starch gel electrophoresis must therefore reside in differences in the charge on the protein molecule at the pH of the gel.

TABLE 4

PROTEINS ISOLATED AND CRYSTALLISED FROM MAMMALIAN SKELETAL MUSCLE SARCOPLASM

| Enzyme | Sedimentation Coefficient $S_{20,w}$ (Svedberg units) | | Molecular Weight | |
|---|---|---|---|---|
| Phosphorylase a | 13.2 | (13) | 495,000 | (13) |
| Phosphorylase b | 8.2 | (13) | 242,000 | (13) |
| Glyceraldehyde -3- P dehydrogenase | 7.0 | (1) | 120,000 | (1) |
| | 7.7 | (2) | 137,000 | (2) |
| Lactate dehydrogenase | 7.0 | (3) | | |
| | 7.4 | (5) | 130,000 | (5) |
| | 6.5 | (4) | | |
| Aldolase | 7.4 | | | |
| | 7.5 | (1) | 149,000 | (1) |
| Enolase | 5.8 | (6) | 64,000 | (7) |
| Creatine phosphokinase | 5.0 | (9,6) | 81,000 | (9,6) |
| Glycerol-1-P dehydrogenase | 4.9 | (8,6) | 78,000 | (8,6) |
| Phosphoglucomutase | 3.7 | (1) | 74,000 | (1) |
| Glycerate P mutase | 3.9 | (6) | 57,000 | (10) |
| | 4.5 | (11) | 64,000 | (11) |
| Adenylate kinase | 2.3 | (12) | 24,000 | (12) |
| Myoglobin | 2.0 | (14) | 17,000 | (14) |

BOVINE SARCOPLASM (EXPERIMENTAL RESULTS)

| | Sedimentation Coefficient $S_{20,w}$ (Svedberg units) | | Sephadex Molecular Weight | |
|---|---|---|---|---|
| $P_1$ | 9.2 | $V_1$ | 150,000 | |
| $P_2$ | 7.7 | $V_2$ | 110,000 | |
| $P_3$ | 5.5 | $V_3$ | 79,000 | |
| $P_4$ | 4.1 | $V_4$ | 52,000 | |
| $P_5$ | 2.0 | $V_5$ | 21,000<br>14,000 | |

Numbers in parentheses refer to references in Table 5.

Table 5. References to Table 4

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Sedimentation coefficients can be used as an index for tentative identification of protein molecules. On the left hand side of Table 4 I have summarised all the available relevant data from the literature on sedimentation coefficients and molecular weights of the sarcoplasmic proteins which have been crystallised from sarcoplasmic extracts. On the right hand side I have summarised our experimental data on bovine sarcoplasm. I have then attempted to correlate the components resolved during our experiments with the published figures for the isolated enzymes. In general it can be seen that the values for sedimentation coefficient and molecular weight fall into five distinct groups; these correlate extremely well with our values for the sedimentation coefficients of the components after gel filtration on G100. The identification is complicated by the fact that most of the published data relates to rabbit muscle sarcoplasmic enzymes; these may not be identical to those found in bovine muscle. There is also the additional complication of possible interactions between the proteins in the sarcoplasmic extracts. The complexity due to polydispersity is partly overcome by the extrapolation of the sedimentation coefficients to infinite dilution. Despite these difficulties the agreement is extremely good.

It seems reasonable to suppose therefore that each of the peaks is composed of proteins of closely similar sedimentation properties (i.e. molecular weight, size and shape) but differing in their enzymic specificity and electrical charge on the protein molecule. The latter is

substantiated by the results of the starch gel electrophoresis of the G100 fractions, which showed that each peak was composed of a few discrete protein components separated into well defined bands.

Myoglobin can be positively identified on the basis of its red colour and also by examination of its spectrum in a recording spectrophotometer as component $V_{5B}$. Scopes (14) has identified several of the protein constituents of sarcoplasm extracts by specific enzymatic staining techniques after separation by starch gel electrophoresis. Comparison of our electropherograms with his separations suggest that both creatine phosphokinase and aldolase occur in the correct G100 fraction. We hope to confirm the presence of the enzymes by using specific enzyme assays on the fractions themselves, and also after separation by electrophoresis.

CONCLUSIONS

Gel filtration of sarcoplasmic extracts on columns offers a satisfactory method for systematically separating the constituent proteins on a preparative scale. It also provides a convenient method for studying changes taking place in the proteins during post mortem period and during the subsequent storage of meat. Information such as changes taking place in the distribution of the proteins can be readily observed, and these can be directly related to changes in the molecular parameters (size, shape and molecular weight) of the proteins as a result of the linear correlation between the elution volume from the Sephadex column and the logarithm of the molecular weight. More strictly the relationship is between the diffusion coefficient and the elution volume - since it is the size and shape of the proteins which determines the separation on Sephadex columns. By further studies on the individual proteins in the Sephadex fractions it should be possible to relate changes in the fractions to changes in individual proteins. In this respect zone electrophoresis of the fraction in starch or polyacrylamide gels followed by specific staining procedures offers a simple and relatively speedy method of following these changes in more detail.

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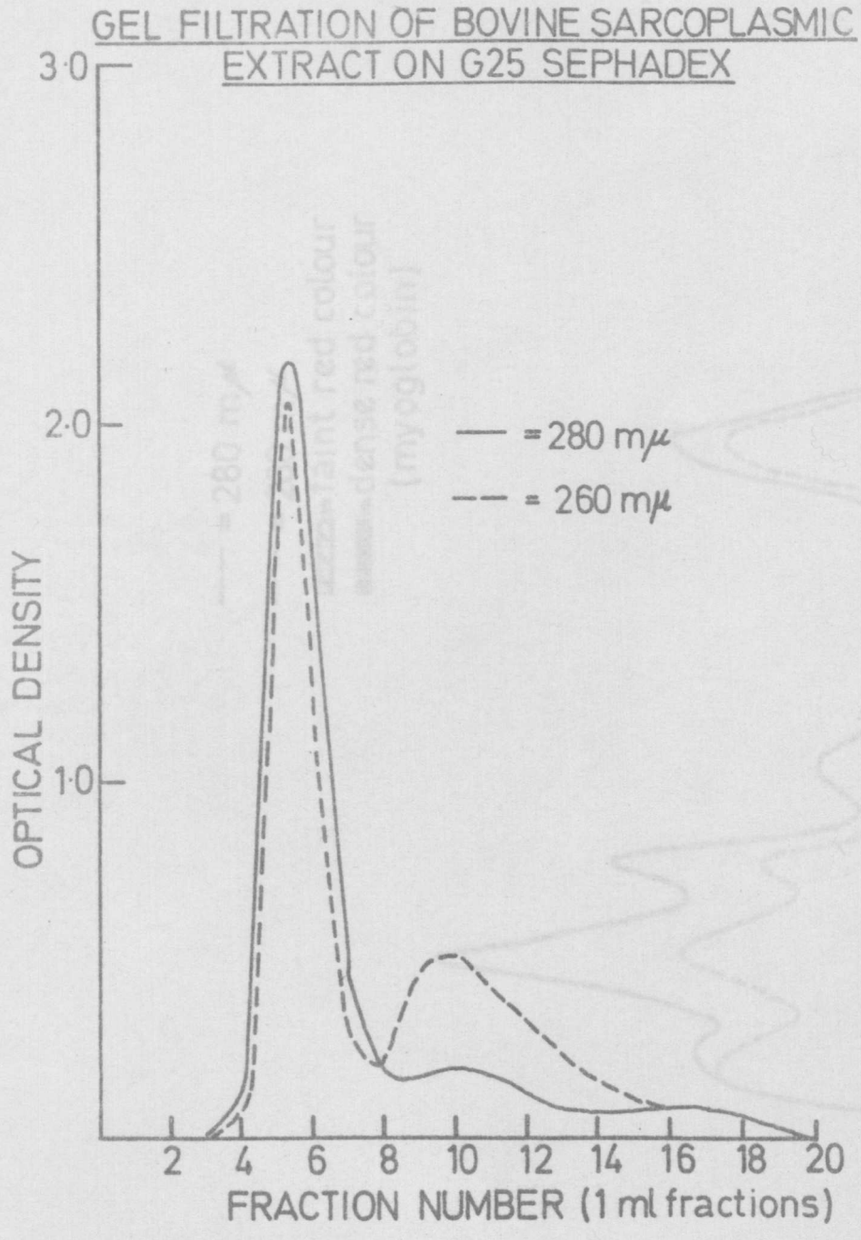
Figure 1


GEL FILTRATION OF BOVINE SARCOPLASMIC EXTRACT ON G25 SEPHADEX
3·0
2·0
1·0
OPTICAL DENSITY
— = 280 mμ
--- = 260 mμ
2
4
6
8
10
12
14
16
18
20
FRACTION NUMBER (1 ml fractions)

GEL FILTRATION OF BOVINE SARCOPLASMIC PROTEINS ON G100 SEPHADEX (150 X 1cm column)

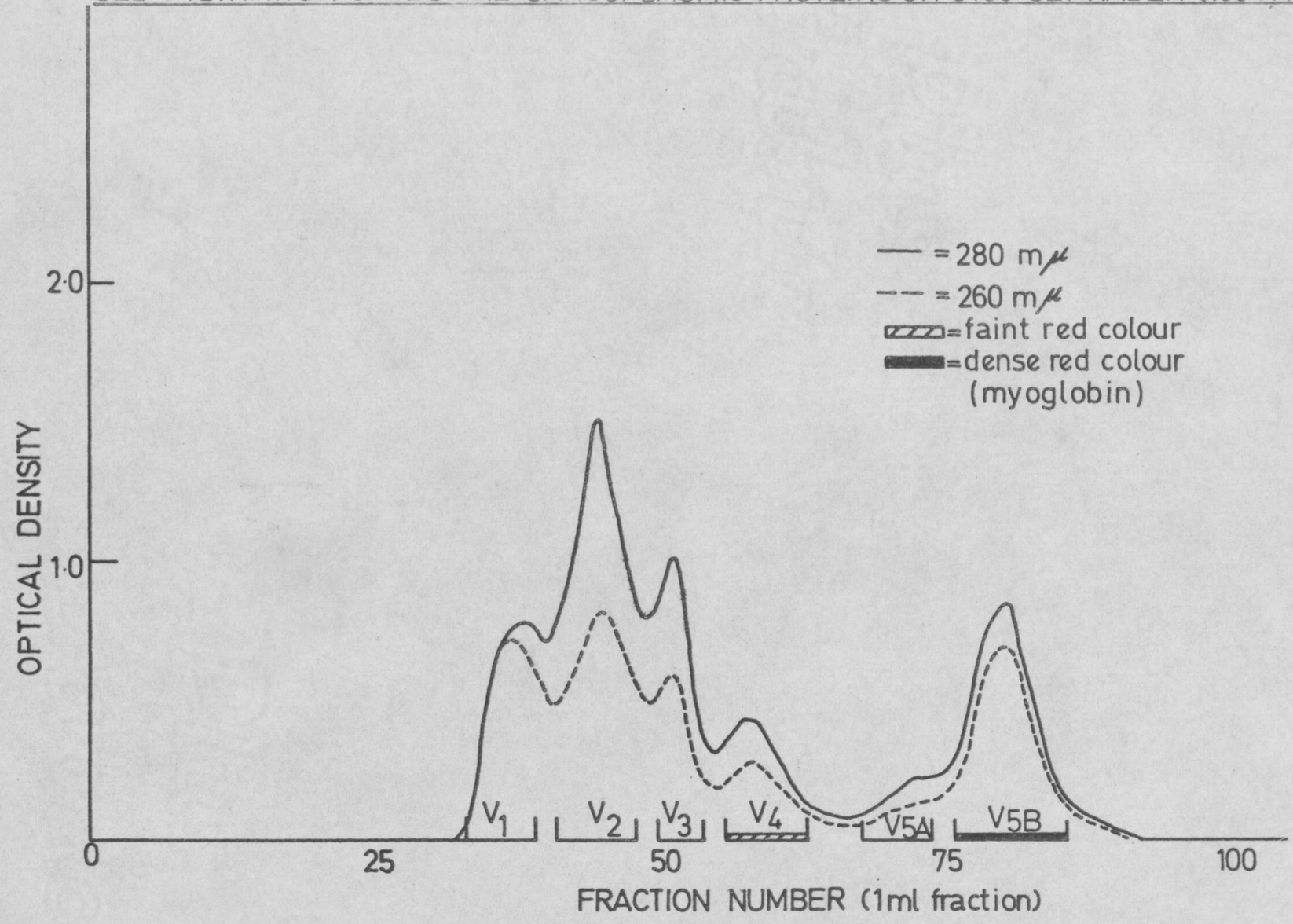



Figure 2